



PATENT APPLICATION

Remarks

It is believed that the specification is now in compliance with 37 CFR 1.821 (d) of the Sequence Rules and Regulations.

No fees are believed due, however, the Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. 01-0519.

Respectfully submitted,

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

Figure 1 (SEQ ID NO: 1) shows the amino acid sequence of human erythropoietin.

Figure 10 (SEQ ID NO: 25) shows the amino acid sequence of the hinge, CH2 and CH3 regions of human IgGy1.

Figure 11 (SEQ ID NO: 26) shows the cDNA and amino acid sequence of Epo N47-Fc fusion polypeptide including the Epo signal sequence. The amino terminal Fc residue is fused to the arg-166 residue of Epo.

... region of an Epo hyperglycosylated analog fusion protein has the sequence as set forth in Figure 10 (SEQ ID NO: 25) (see Ellison *et al.*, Nucleic Acids Res. 10, 4071-4079 (1982)) starting at residue 6 (that is, residues 1-5 are deleted).

Cysteine residues in Fc molecules can be deleted or replaced with other amino acids to prevent formation of disulfide crosslinks. In particular, a cysteine residue at position 5 of Figure 10 (SEQ. ID. NO. 25) may be substituted with one or more amino ...

An Fc fragment may be prepared by deletion of one or more amino acids at any of positions 1, 2, 3, 4 and 5 as shown in Figure 10 (SEQ ID NO. 25). In one embodiment, the amino acid residues at positions 1-5 inclusive are deleted. Substitutions at these positions can also be made and are within the scope of this invention.

The Fc protein may be also linked to the Epo glycosylation analogs by "linker" moieties comprising chemical groups or amino acids of varying lengths. Such chemical linkers are well known in the art. Amino acid linker sequences can include but are not limited to:

- (a) ala-ala-ala;
- (b) ala-ala-ala-ala (SEQ ID NO: 6);
- (c) ala-ala-ala-ala-ala (SEQ ID NO: 7);
- (d) gly-gly;
- (e) gly-gly-gly;
- (f) gly-gly-gly-gly-gly (SEQ ID NO: 8);
- (g) gly-gly-gly-gly-gly-gly-gly (SEQ ID NO: 9);
- (h) gly-pro-gly;
- (i) gly-gly-pro-gly-gly (SEQ ID NO: 10); and
- (k) any combination of subparts (a) through (i).

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For each analog, the same outside primers were used. The 3' (reverse) primer contained sequences that introduced a stop codon followed by a Sal I restriction site:

AGGTGGACAGTCGACATTATCTGTCCCCTGTC (SEQ ID NO: 11).

The 5' forward reaction primer:

AACAAGCTTCTAGACCACCATGGGGGTG (SEQ ID NO: 12)

had a Hind III restriction site followed by a Kozak sequence upstream of the Epo initiator codon (ATG).

Mutagenic primers were as follows:

N30 T32 mutagenic forward primer

ACG ACG GGC TGT AAT GAA ACG TGC AGC TTG (SEQ ID NO: 13)

N30 T32 mutagenic reverse primer

CAA GCT GCA CGT TTC ATT ACA GCC CGT CGT G (SEQ ID NO: 14)

N55 T57 mutagenic forward primer

GCC TGG AAG AGG ATG AAT GTC ACGCAG CAG GCC GTA GAA (SEQ ID NO: 15)

N55 T57 mutagenic reverse primer

TTC TAC GGC CTG CTG CGT GAC ATTCAT CCT CTT CCA GGC A (SEQ ID NO: 16)

V87 N88 T90 mutagenic forward primer

TCT TCC CAG GTG AAT GAG ACC CTG CAG CTG (SEQ ID NO: 17)

V87 N88 T90 mutagenic reverse primer

CAG CTG CAG GGT CTC ATT CAC CTG GGA AGA GTT G (SEQ ID NO: 18)

P124 T125 T126 mutagenic forward primer

CCA GAT CCG ACC ACA GCT GCT CCA (SEQ ID NO: 19)

P124 T125 T126 mutagenic reverse primer

TGG AGC AGC TGT GGT CGG ATC TGG A (SEQ ID NO: 20)

Epo analog N70 was also made by overlap PCR. Plasmid DSR*2 containing the cDNA sequence encoding analog N47 (N30 T32 V87 N88 T90) and plasmid pAMG21 (ATCC accession no. 98113) containing cDNA encoding an Fc region were used as templates for the polymerase chain reactions. The Fc portion of human immunoglobulin IgG1 heavy chain from residue 104 of the hinge domain(Asp-104) to the carboxyl terminus (Ellison et al., *supra*, see also Figure 10 starting at aspartic acid residue at position 6), was generated by PCR amplification of a human spleen cDNA library (Clontech). Overlapping PCR products were generated in two reactions using the following oligonucleotide primers

5' forward reaction primer 2343-85 (Epo specific):

AAC AAG CTT CTA GAC CAC CAT GGG GGT G (SEQ ID NO: 21)

3' reverse reaction primer 2343-87 (homology to both Epo and Fc):

AGG TGG ACA TGT GTG AGT TTT GTC TCT GTC CCC TCT CCT GCA GGC CTC C (SEQ ID NO: 22)

5' forward reaction primer 2343-86 (homology to both Epo and Fc):

GAG GCC TGC AGG ACA GGG GAC AGA GAC AAA ACT CAC ACA TGT CCA CCT (SEQ ID NO: 23)

3' reverse reaction primer 2343-88 (specific to Fc):

TGG ACA GTC GAC ATT ATT TAC CCG GAG ACA GGG AGA GGC TCT TCT GC (SEQ ID NO: 24)

44. The fusion protein of Claim 43 consisting of the mature amino acid sequence as set forth in Figure 11 (SEQ ID NO: 26) lacking the signal sequence.